A Rapid Screening Test for Pyroluria; Useful in Distinguishing a Schizophrenic Subpopulation

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An assay procedure is presented which has proven extremely useful in delineating a subpopulation of schizophrenic patients. The procedure is a highly sensitive rapid screening test for urinary pyrroles such as kryptopyrrole and urobilinogen. An extensive investigation of this test and the original assay procedure for kryptopyrrole described by Irvine indicates that free kryptopyrrole arises primarily as an artifact of the original procedure and that it exists ordinarily in a bound form in the urine.

The patient population described as excreting kryptopyrrole probably suffers from a general pyroluria where elevated amounts of pyrrole metabolites such as kryptopyrrole, urobilinogen, and urobilinoid pigments can be detected in the urine. The presence of these compounds may lead to a zinc deficiency, and possibly also to a deficiency in vitamin B6, which results in the most severe clinical symptoms exhibited by these patients.

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INTRODUCTION

The association of the mauve factor or kryptopyrrole in urine with certain forms of psychosis has been reported and confirmed by several investigators (Irvine, 1961; Hoffer and Mahon, 1967; Sohler et al., 1967; Huszak et al., 1972). Recently, Irvine (1974) has shown that the multiple spot formation observed in this assay is partly an artifact of the chromatographic assay employed and that kryptopyrrole exists primarily as an oxygenated lactam form. From a practical viewpoint, the observation that patients who are mauve positive respond in many instances to combined treatment with zinc and Pyridoxine is very significant (Pfeiffer and Iliev, 1973). Most patients with acute intermittent porphyria (AIP) excrete kryptopyrrole (Huszak et al., 1972). A likely endogenous source of kryptopyrrole is bile pigment, although an aberrant synthetic pathway from porphyrins cannot be ruled out. However, direct experimental evidence for this at the moment is lacking, and it is not known

how this metabolite is synthesized. Furthermore, there are questions as to whether the assay procedure employed is actually measuring kryptopyrrole or some other pyrrole metabolite.

The present report concerns itself with an examination of two assay procedures that have been employed for determining what is believed to be kryptopyrrole. Both of these procedures have delineated the same kind of a patient population. The two procedures are the charcoal absorption method originally described by Irvine and a simpler method involving chloroform extraction and subsequent color development with Ehrlich's reagent.

MATERIALS AND METHODS

Since the chloroform extraction, Ehrlich reaction procedure has not previously been published, we present in detail as follows:

Preparation of Reagents

(1) Ehrlich Reagent

One gram of p-dimethylaminoben-zaldehyde is dissolved in about 80 ml of methanol in a 100 ml volumetric flask. The flask is cooled in an ice bath, and 5 ml of concentrated H_2SO_4 is added slowly to prevent excessive heating. After the acid addition, the flask is allowed to reach room temperature, and the solution is adjusted to a volume of 100 ml with methanol. The solution is stored in a brown bottle, refrigerated, and it is stable for three days.

(2) Mcllvaine Citrate Phosphate Buffer

Mix 15.89 ml of 0.1 M citric acid with 8.11 ml of 0.2 M dibasic sodium phosphate. The final pH should be pH-3.0.

(3) Kryptopyrrole Standard

Synthetic kryptopyrrole was obtained from Aldrich Chemical Co., Inc.

Storage of Pure Kryptopyrrole

Since kryptopyrrole is extremely sensitive to oxidation by air, it is necessary to divide the 5 gms of kryptopyrrole into approximately 100 - 200 mg aliquots and store them under nitrogen in sealed glass ampules. This operation requires at least two people. One person takes a set of 1 or 2 ml ampules and flushes them with a stream of nitrogen. The stock vial is opened, and 20 drops of kryptopyrrole is placed into each ampule with a Pasteur pipet. One person then flushes, each ampule with nitrogen while the other person seals the ampule in a flame. The ampules are stored in the dark at -20°C.

Preparation of Stock Standard

A 200 ug/ml stock solution of kryptopyrrole is prepared by weighing 20 mgs (about 2 drops from a Pasteur pipet) of pure kryptopyrrole into a 5 or 10 ml beaker. The kryptopyrrole is transferred quantitatively from the beaker to a 100 ml volumetric flask with a small amount of methanol (less than 5 ml). The solution is brought to volume with 1 percent ascorbic acid. This stock standard is stable for about 24 hours if aliquots are stored frozen.

Preparation of Working Standard

The stock standard is diluted one to 10 with 1 percent ascorbic acid. This solution is made up fresh each time, and a standard curve is set up in the range of 10g to 15ug.

Urine Assay Procedure

Urine is collected in a container containing "Vitamin C," 500 mg.

A 2 ml urine sample is placed in a glass stoppered centrifuge tube," and the pH is adjusted to between 3 and 4 by either the addition of 0.I N HCI or by the addition of 2 ml of citrate phosphate buffer (pH-3). The urine is then extracted with 4 ml chloroform by shaking either by hand or on a Vortex shaker for about two minutes and centrifuged to break up any emulsion. After centrifugation the top aqueous layer is carefully removed, and 100 - 200 mg anhydrous sodium sulfate is added to the chloroform and shaken briefly to remove traces of aqueous globules.

Two ml of the clear chloroform extract are placed in a clean test tube and 0.5 ml

of Ehrlich's reagent is added and shaken briefly. After 30 minutes the intensity of the color is read in a spectrophotomer at 540 nm. The normal range is 0 - 20 ug percent — patients may read as high as 200 - 400ug percent.

Calculations

ug of unknown (obtained from the calibration curve) X50 = *ug* kryptopyrrole/100 ml urine.

The charcoal procedure was carried out as originally described by Irvine, 1961. Spectral studies were carried out on a Bausch and Lomb, Spectronic 505.

Chromatographic Studies

In addition to the isopropanokam-monia: water 20:1:2 system originally described by Irvine, the following TLC systems on Quanta silica gel plates Q5 or

PQ5-500 (preparative) were employed:

(1) methyl acetate: isopropanol: 25 percentammonium hydroxide (45:35:20)

(2) chloroform: 96 percent acid (95:5)

(3) cumene: ethyl acetate: ethanol (8:7:1)

Tanks were equilibrated prior to use.

Samples were dissolved in chloroform or methanol and 3 - 10 ul were applied generally, but for preparative purposes 0.5 - 1 ml of sample was applied. Detection of compounds on the plate was carried out by examination under UV light, spraying with Ehrlich's reagent, and by spraying with iodine and zinc with subsequent examination for fluorescence under UV light.

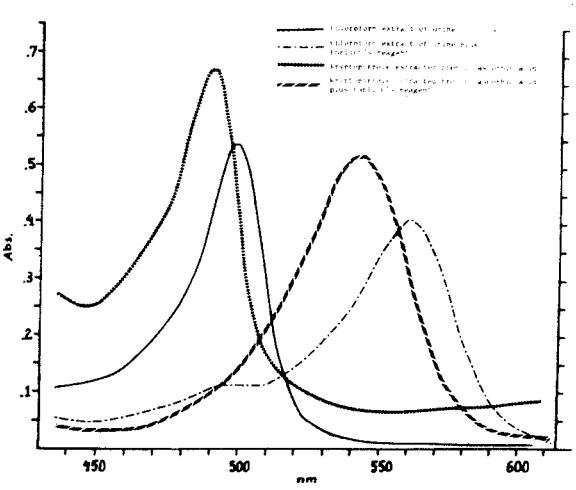
Elution of materials from preparative plates was carried out by removing a particular band and extracting it five times with a ml of methanol.

RESULTS AND DISCUSSION

Our initial studies were carried out using the procedure described by Irvine as at that time the mauve factor was operationally defined. The procedure was quite time consuming and at best semiquantitative. In addition to this, evaluation of the mauve-colored spot was quite subjective. The chloroform extraction procedure was developed primarily as a rapid preliminary screening procedure which could be followed by methods having greater specificity. Kryptopyrrole is quantitatively extracted with chloroform, and with Ehrlich's reagent it gives a chromophore with an absorption maximum of 540 nm. Using this procedure a population of schizophrenic patients was defined as being mauve positive, who in many instances responded to treatment with adequate Pyridoxine and zinc.

Because of the known lack of specificity of the Ehrlich reaction, it was decided to examine the chloroform extract of urine more closely as to the nature of the compounds which might be Ehrlich reacting materials. An examination of the chloroform extract from a number of patients indicated that the principal constituent which was found consistently had a maximum at 497 nm and gave an Ehrlich reaction chromophore with a maximum at 559 nm. This was in contrast to the peak observed in acid media of kryptopyrrole which had a maximum at 482 nm and gave an Ehrlich chromophore with a peak at 540 nm.

The Ehrlich reacting material in urine, therefore, appears to be distinct from kryptopyrrole. An examination of pyrrole metabolites which can be found as urinary constituents indicated the material being measured in the chloroform extract was primarily urobilinogen. Figure 1



Comparison of spectra of urinary material with kryptopyrrole prior to and after reaction with Ehrlich's reagent.

TABLE 1

Chromatographic Behavior of Kryptopyrrole and Urinary Pyrrole Metabolites

Sample	Isopropanol ammonia water (20:1:2) (paper)	Isopropanol Methyl Acetate Ammonia (45:35:20) (Silica Gel)	Chloroform 95% Acetic Acid (95:5) (Silica Gel)	Cumene Ethyl Acetate Ethanol (8:7:1) (Silica Gel)
	rf	rf	rf	rf
Kryptopyrrole	.90	.89	.73	.92
Urobilinogen	.19	.26	.38	.15 (tailing)
Urobilin	.07	.18	0	.0
Urinary fraction 2	.05	.18	0	0
Urinary fraction 3	.19	.27	.37	.15 (tailing)

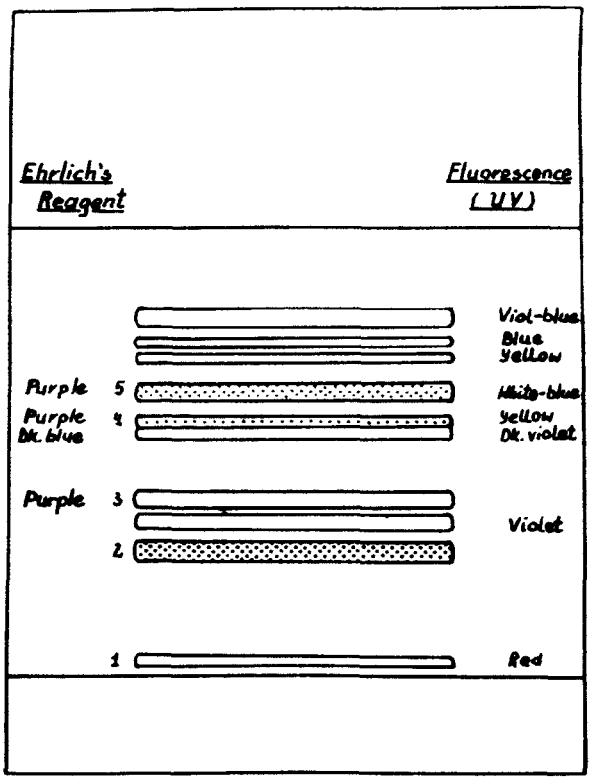
Figure 1 gives the absorption spectra of the Ehrlich reaction products obtained with urobilinogen, kryptopyrrole, and the chloroform extract obtained from urine. The urinary chloroform extract behaves identically with urobilinogen.

The nature of the material in the urine extract was subsequently confirmed by thin layer chromatography Table 1 gives the Rf values of kryptopyrrole, urobilinogen, and urobilin as well as fractions obtained from urine in several solvent systems and indicated that most of the material in the urine reacting with Ehrlich's reagent is urobilinogen.

Figure 2 is of a preparative plate of a chloroform extract prepared from about 100 - 150 ml of urine from a mauve-positive patient. The principal Ehrlich reacting material is band three which behaves identically with urobilinogen, prepared by the reduction of bilirubin with sodium amalgam. Band two is urobilin, as this material arises by oxidation of urobilinogen. The material found in band two upon addition of Zn gives a green fluorescence which is characteristic of urobilin. Band three material upon oxidation with iodine and addition of Zn yields the same green fluorescent product. The band designated as band one is a porphyrin; it was characterized by a bright red fluorescence and an absorption spectrum which

is characteristic of a porphyrin. On occasion two such red fluorescent bands have been observed on or near the origin.

Figure 2



Principal band detected on a preparative plate of a chloroform extract of urine from a mauve-positive patient. The bands number 1 - 5 are believed to represent pyrrole metabolites; Band (1) a porphyrin, (2) urobilin, (3) urobilinogen (4 and 5) unidentified metabolites related to urobilinoid pigments of kryptopyrrole. Dotted bands can be detected by color under visible light.

Among the Erhlich reacting compounds observed on the plate is 3-indole acetic acid. This is located in the band immediately below band number 4. Although it reacts with Ehrlich's reagent, it reacts at a much slower rate than urobilinogen, and it appears not to interfere with the assay procedure normally.

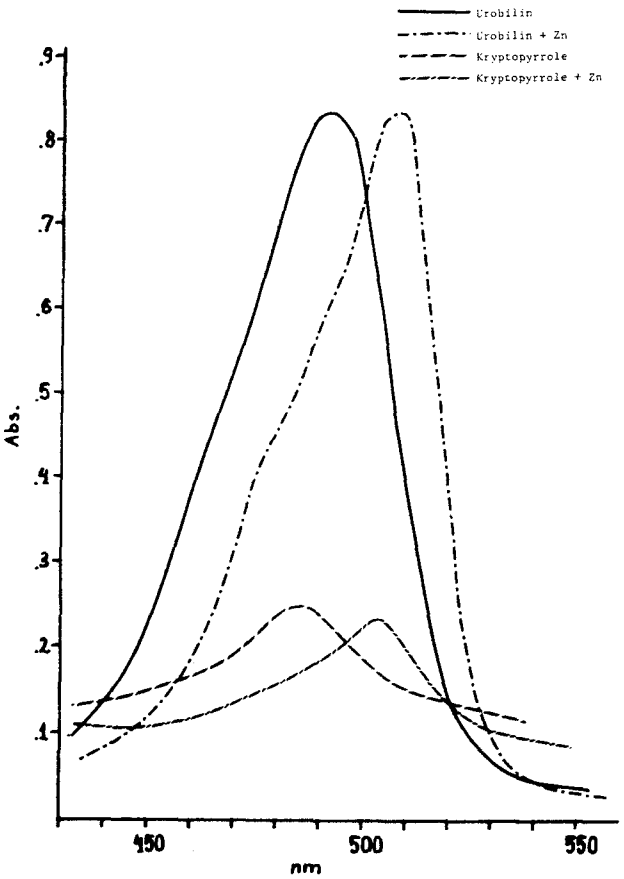
Bands four and five, we believe, represent at least in part metabolites related to the urobilinoid pigments or possibly the lactam of kryptopyrrole. These bands are characterized as reacting weakly with Ehrlich's reagent and forming the chromophore much more slowly than urobilinogen. On rechromatography of bands four and five in other systems both bands are found to be complex mixtures. Whether some of the material in this band represent dipyrroles or pyrroles with altered functional groups has not been resolved.

It is also noted that we do not find a band corresponding to synthetic kryptopyrrole. This compound moves in this system very close to the solvent front, Rf - 0.89. Both on the basis of spectral and chromatographic evidence, free kryptopyrrole could not be detected.

If free kryptopyrrole cannot be detected in the urine, how does it arise in the charcoal procedure of Irvine? In order to answer this question, preparative TLC chromatography was carried out on a chloroform extract of urine. Fractions (as illustrated in Figure 2) corresponding to porphyrins, urobilin, urobilinogen as well as fractions 4 and 5, the faster moving components, were eluted. These fractions were subsequently taken up in 1 percent ascorbic acid and carried through the charcoal procedure. The urobilin, urobilinogen, and fractions 4 and 5 yielded material which corresponded to kryptopyrrole on chromatography. When the fractions were tested without passage on charcoal, no kryptopyrrole was found. Fractions 4 and 5 yielded kryptopyrrole most readily. From these results, it would appear that free kryptopyrrole arises from the dissociation of some bound forms during the course of the charcoal procedure.

A similarity of behavior is evident between naturally occurring urobilinoid pigments and kryptopyrrole. Both kryptopyrrole and urobilinogen, on standing in an acid medium, undergo an auto-oxidation and result in products having distinct absorption spectra at about 480 - 500 nm. The auto-oxidation is quite rapid in both instances, particularly in the presence of light. Both compounds react with Ehrlich's reagent in solution in a similar manner giving rapidly a mauve color. Both compounds, particularly on oxidation, appear to form complexes with metals such as Zn and also with compounds such as Pyridoxine. The formation of a Zn complex could be detected spectrophotometrically with both urobilin and kryptopyrrole (Figure 3). In both instances the complex was dissociated on acidification. In addition,

Figure 3



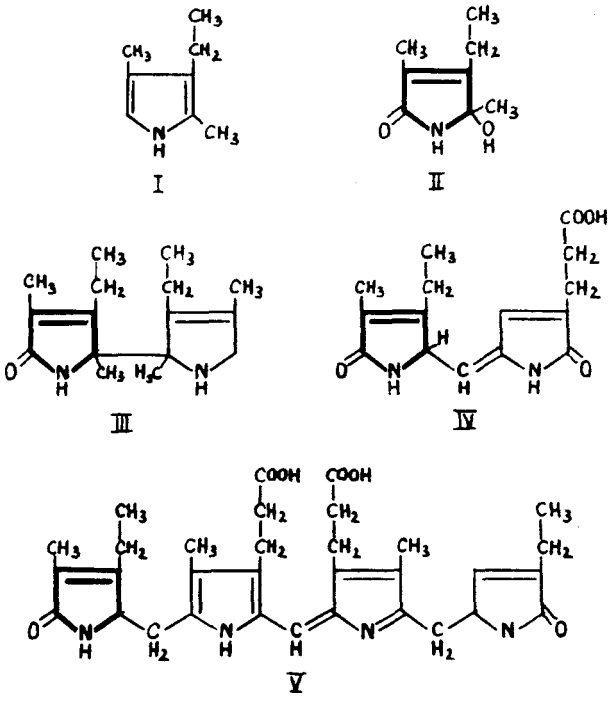
Urobilin and kryptopyrrole Zn complex formation.

we have some evidence that kryptopyrrole may interact with urobilinoid pigments, possibly by some kind of adduct formation.

The similarity in behavior is most likely due to their lactam structure. This similarity is illustrated in Figure 4.

Comparison of the structures of kryptopyrrole and related compounds with urobilinoid pigments. I. kryptopyrrole. II. a lactam form of kryptopyrrole. III. a dipyrrole oxidation product of kryptopyrrole. IV. bilifuscin. V. urobilin.

Figure 4



The ring corresponding to the lactam structure of kryptopyrrole is darkened.

Kryptopyrrole (I) is auto-oxidizable, particularly in the presence of light resulting in an oxygen at position five, forming typical lactam structures (II and III).

The dimer (III) was first reported by Hoft et al. (1967) as a product of auto-oxidation. Recently Lightner et al. (1973) studied the dye-sensitized photo-oxidation of kryptopyrrole and found a number of isomeric forms among which was compound II.

A similar structure is also found in the naturally occurring urobilinoid pigments bilifuscin (IV) and in urobilin(V). It has been shown that such structures tend to form adducts by hydrogen bonding, and, in the case of the tetrapyrrole compounds, it is believed that the structure is held in a ring form similar to the porphyrins by hydrogen bonding. In urobilin the outer pyrrole rings of the tetrapyrrole chain are believed to occur primarily as a lactam. In fact, one of the outer rings of urobilin, if it were split from the chain, would be kryptopyrrole.

Kryptopyrrole has also been shown to complex Zn and interact with Pyridoxine. This type of behavior may explain the clinical observation of some schizophrenic patients appearing to suffer from a Zn and Pyridoxine deficiency, although it is conceivable that the Pyridoxine deficiency could be the result of a Zn deficiency in that the active form of Pyridoxine is the phosphate, the phosphorylation of which is carried out by a kinase which requires Zn for activity. Although we find no evidence for free kryptopyrrole, the possibility of it existing in a bound or in the form of a complex cannot be ruled out, and indeed the evidence seems to indicate this is the case.

The assay described is an extremely sensitive assay for urobilinogen. It can also measure any free kryptopyrrole. It is evident that the patients that are designated as being mauve positive are excreting increased levels of a number of pyrrole metabolites both free and bound. Most of the patients have elevated levels of urobilinogen, kryptopyrrole in a bound form, and, in some cases, even coproporphyrin levels are elevated. This generalized pyroluria, we believe, may be due to a stress-induced anomaly of intestinal permeability which permits these pyrroles to get into the systemic circulation.

The use of kryptopyrrole as a standard in the assay procedure requires further comment. The instability of urobilinogen is such that it is almost impossible to use it as a standard. Kryptopyrrole serves as a more convenient standard for both itself and urobilinogen on account of its greater stability and the fact that the chromophore maxima for both of these compounds are close enough to be usable, with reasonable sensitivity.

The utility of this method in delineating a subpopulation of schizophrenic patients has already been mentioned. The patients represent some 30 percent of the total schizophrenic population, and these patients represent several distinguishing clinical features, namely white spots in fingernails, left upper quadrant pain, dysperceptive schizophrenia with fairly good affect, failure to remember dreams, and a sweetish breath odor. These patients respond to treatment with fairly large doses of B6 and zinc supplement.

In conclusion, it would appear that kryptopyrrole does not occur free in the urine, but may occur in a bound form or in the form of an adduct. Kryptopyrrole arises in the charcoal procedure of Irvine, primarily as an artifact of the procedure possible by dissociation of a bound form. Urobilinogen is the primary Ehrlich reacting material found in the urine of patients characterized as mauve positive. The urobilinogen levels are in the range of 50 - 300ug/%.

It appears that mauve-positive schizophrenic patients suffer from a condition where somewhat elevated amounts of pyrrole metabolites get into the systemic circulation. This pyrolemia may result in a zinc deficiency due to the formation and excretion of pyrrole zinc complexes.

REFERENCES

HOFFER, A., and MAHON, M.: The presence of unidentified substances in the urine of psychiatric patients. J. Neuropsychiat. 2:331-367, 1961.

HOFT, E., KATRITZKY, A.R., and NESBIT, M.R.: The auto-oxidation of alkyl pyrroles. Tetrahedron Letters 32:3041-3044, 1967.

HUSZAK, I., DURKO, I., and KARSAI, K.: Experimental data to the pathogenesis of kryptopyrrole excretion in schizophrenia. Acta Physiol, Acad. Scientiarium Hungar-icza, 42:37-86, 1972.

IRVINE, D.G.: Apparently non-idolic Ehrlich-positive substances related to mental illnesses. J. Neuropsychiat. 2:292-305, 1961.

IRVINE, D.G.: Kryptopyrrole and other monopyrroles in molecular neurobiology. Int. Rev. Neurobiol. 17:145-182, 1974.

JACKSON, A.H., SMITH, K.M., GRAY, C.H., and NICHOLSON, D.C.: Molecular species of the urobilins. Nature 209:581-583, 1966.

LIGHTNER, D.A., and CRANDALL, D.C.: The dye sensitizer photo oxygenation of kryptopyrrole. Experentia 29:262-264, 1973.

PFEIFFER, C.C., and ILIEV, V.: Pyroluria Urinary Mauve Factor Causes Double Deficiency of Bg and Zinc in Schizophrenics. Fed. Proc. 32:276, 1973.

SOHLER, A., RENZ, R.H., SMITH, S., and KAUFMAN, J.: Significance of hydroxyskatole and Mauve Factor Excretion in Schizophrenia. Int. J. Neuropsychiat. 3:327-331, 1967.